Recombinant Soluble Form of PSGL-1 Accelerates Thrombolysis and Prevents Reocclusion in a Porcine Model

Anjali Kumar, PhD; Mario P. Villani; Umesh K. Patel, BS; James C. Keith, Jr, DVM, PhD; Robert G. Schaub, PhD

Background—We investigated whether administration of a soluble recombinant P-selectin glycoprotein ligand-1 chimera (rPSGL-Ig) in conjunction with thrombolytic therapy would enhance thrombolysis by preventing ongoing interactions of leukocytes with platelets and the injured arterial wall.

Methods and Results—An occlusive thrombus was formed in an internal iliac artery of Yorkshire pigs by placement of a copper coil in the artery under fluoroscopic guidance. Pigs then received heparin and, 15 minutes later, either vehicle or rPSGL-Ig followed by infusion with 25 mg tissue plasminogen activator according to the 90-minute regimen. Blood flow through the artery was monitored by angiography and scored on a scale of 0 to 3. Lysis of the thrombus was accelerated by 70% in pigs treated with rPSGL-Ig 250 μg/kg compared with control (13.3 ± 5.0 versus 44.4 ± 13.3 minutes; n = 9 each). Eight of 9 control pigs reoccluded in 13.8 ± 16.9 minutes after the end of tissue plasminogen activator infusion, whereas no reocclusion was observed in 8 of 9 pigs in the rPSGL-Ig group. When the dose of rPSGL-Ig was increased to 500 μg/kg, time to lysis was shortened by 61% from control (18.0 ± 8.4 versus 46.0 ± 8.9 minutes). Reoocclusion occurred in 6.0 ± 15.2 minutes in control but not in any rPSGL-Ig–treated pig (n = 5 each). In addition, near-normal flow (score 2 or 3) after thrombolysis was achieved 59% and 58% faster in the 2 rPSGL-Ig groups than in their respective controls.

Conclusions—Inhibition of leukocyte accumulation at the site of thrombosis with rPSGL-Ig may represent a safe therapeutic intervention that could be important in accelerating thrombolysis, achieving optimal reperfusion, and reducing incidence of acute reocclusion. (Circulation. 1999;99:1363-1369.)

Key Words: thrombolysis ■ fibrinolysis ■ thrombosis ■ cell adhesion molecules ■ platelets ■ leukocytes

The association of acute myocardial infarction with thrombus formation, frequently subsequent to plaque rupture,1,2 has made early thrombolytic therapy the standard of care for patients.3 Early initiation of thrombolytic therapy after the onset of symptoms reduces myocardial necrosis, preserves ventricular function, and decreases morbidity and mortality.4–6 However, with current thrombolytic therapy regimens, only ≈25% of all treated patients have timely, adequate, and sustained coronary reperfusion.7 Hence, efforts have been directed toward developing adjunctive therapies that can enhance the thrombolytic effect of currently available agents. Inhibitors of platelet αIIbβ3 (GP IIb/IIIa) integrin receptors have received much attention, and several inhibitors have been studied in various models of thrombosis. Notably, the murine antiplatelet antibody 7E3 in conjunction with tissue plasminogen activator (tPA) was shown to accelerate thrombolysis and prevent reocclusion in animal studies.8 More recently, results presented from the Thombolysis in Myocardial Infarction (TIMI) 14 clinical trial suggest that combination of ReoPro (chimeric 7E3 Fab; abciximab) with reduced doses of tPA or streptokinase enhanced thrombolysis.9 However, significant issues, such as bleeding and thrombocytopenia, still remain with compounds of this class, especially in combination with heparin, and a therapeutic with a better safety profile will be of value in the clinic. P-selectin is a membrane glycoprotein adhesion molecule contained within platelet α-granules10,11 and Weibel-Palade bodies of endothelial cells12,13 that is rapidly mobilized to the plasma membrane on cell activation and granule secretion. It is a member of the selectin family, which also includes E-selectin and L-selectin. Selectins mediate rapid transient interactions of leukocytes “rolling” on endothelial cells, generally believed to be the prerequisite for firm adhesion and subsequent extravasation.14 In addition, P-selectin mediates adherence of activated platelets to leukocytes, such as monocytes and neutrophils.15,16 The high-affinity counterreceptor for P-selectin is a mucin-like glycoprotein, P-selectin glycoprotein ligand-1 (PSGL-1), expressed on the surface of myeloid cells.17,18 Platelet-leukocyte interaction is of considerable pathophysiological interest because it not only serves to target both cell types to appropriate sites of inflammation and/or hemostasis but also causes further functional alteration in both cell types.
Platelet-leukocyte interactions, mediated by P-selectin, have been implicated in thrombus amplification and stabilization. In a key report by Palabrica et al., leukocyte accumulation and fibrin deposition in thrombi were found to be P-selectin–dependent. Adhesion of monocytes and platelets via P-selectin may cause tissue factor release from monocytes, which can initiate further coagulation and conversion of fibrinogen to fibrin. Interaction of platelets and neutrophils leads to the release of cathepsin G from neutrophils, which can damage endothelium and expose the subendothelial matrix and may attract further platelet adhesion.

Thrombi formed in the presence of an anti–P-selectin antibody lysed more rapidly on subsequent thrombolytic therapy in a cynomolgus monkey model, likely because of reduced fibrin content of the thrombi. In the present study, we investigated whether administration of a recombinant soluble PSGL-1 chimera (rPSGL-Ig) at the time of thrombolytic therapy would enhance fibrinolysis by preventing ongoing interactions of leukocytes with platelets and the injured arterial wall.

**Methods**

**rPSGL-Ig Chimera**

PSGL-1 is the physiological ligand for P-selectin and binds P-selectin via an anionic amino-terminal peptide sequence. rPSGL-Ig is produced in Chinese hamster ovary cells, which have been engineered to coexpress the critical carbohydrate-modifying enzymes fucosyltransferase VII and core2 GlcNAc transferase. It comprises the first 47 amino acids from the N-terminal end of the extracellular domain of mature PSGL-1, fused at the “hinge” region of human IgG1. Two “hinge-proximal” amino acids at positions 234 and 237 within the IgGFc portion are mutated to alanine to reduce complement activation and Fc receptor binding. This manipulation of the compound also gives it a long half-life.

**Animal Procedures**

All animals used in this study were handled in compliance with the Guide for the Care and Use of Laboratory Animals, 1996, a publication of the National Research Council, National Academy Press, Washington, DC. Experiments were performed in an American Association for Accreditation of Laboratory Animal Care–approved laboratory according to protocols that were reviewed and approved by the Institutional Animal Care and Use Committee.

**Induction of Arterial Thrombosis**

Twenty-eight Yorkshire pigs (10 kg), male and female, were anesthetized with tiletamine and zolazepam 6.6 mg/kg and xylazine 4.4 mg/kg IM. Surgical anesthesia was maintained by inhalation of 1.25% to 2.5% isoflurane (Inhalant Isoflurane Vaporizer System, Ohio Medical Products). The animals were allowed to respire on 1.5 to 2.5 L/min oxygen. Fluids and drugs were administered via the marginal ear veins of both ears. A 5F pediatric catheter (Cordis Corp) was introduced through the left femoral vein into the inferior vena cava for blood sampling. A 7F introducer (CR Bard) was placed in the left common carotid artery and used to position a Teflon-coated guidewire (0.014 in) into the left or right internal iliac artery under fluoroscopic guidance. A copper coil, made by wrapping a 26-gauge wire tightly around a 20-gauge needle, was placed over the guidewire and directed into the internal iliac artery with a 7F percutaneous catheter (CR Bard). Once the coil was positioned, the guidewire was removed and the catheter withdrawn into the abdominal aorta and used for the injection of contrast medium (diatrizoate meglumine and diatrizoate sodium; Mallinckrodt Medical) for arteriograms.

An occlusive thrombus was formed within minutes of placement of the copper coil, as judged by angiography. All animals were given 2000 U heparin IV, followed by an infusion of 1500 U/h in 0.9% saline to prevent clot extension. Heparin infusion was continued throughout the experiment and was adjusted to keep activated clotting time (measured every 15 minutes) at 2-fold to 3-fold of baseline.

**Drug Administration and Flow Monitoring**

After the thrombus had been aged for 15 minutes, an angiogram was taken to confirm vascular occlusion. Animals then received a bolus of either rPSGL-Ig 250 or 500 µg/kg IV or vehicle (rPSGL-Ig formulation buffer) followed by human recombinant tPA (Activase; Genentech, Inc). All pigs received 25 mg tPA at 1 mg/mL infused intravenously over 90 minutes via a syringe pump (Harvard Apparatus, Inc), with 10% of the dose delivered as an initial bolus immediately after the rPSGL-Ig or vehicle bolus. Arteriograms were taken every 10 minutes for up to 60 minutes after the end of tPA infusion and were recorded for later playback and confirmation of results. The times taken for lysis of the thrombus and reocclusion of the artery were recorded. The experiment was terminated at reocclusion or at 60 minutes after the end of tPA infusion, whichever came first.

**Scoring of Quality of Flow Reestablished**

Blood flow reestablished past the thrombus in the copper coil on treatment with rPSGL-Ig or vehicle and tPA was scored as 0, 1, 2, or 3, with 0 indicating an occluded vessel and 3 indicating “normal” flow. Time taken for reestablishment of “near-normal” flow (score of 2 or 3) was compared.

**Blood Sampling**

Blood samples were obtained from the inferior vena cava at baseline, before rPSGL-Ig/vehicle and tPA administration, at reperfusion, and at termination for the following tests, performed by Colorado Coagulation Consultants: activated partial thromboplastin time, fibrinogen, D-dimer, platelet count, and plasminogen activator inhibitor-1 (PAI-1) antigen, PAI antigen, and PAI activity. Assays for other tests were not available for porcine samples. In addition, for rPSGL-Ig–treated animals, blood samples were drawn at baseline, 1 minute after drug administration, and at termination to determine serum levels of rPSGL-Ig with an ELISA.

**Data Analysis**

Data are presented as mean±SD. Hypothesis testing for differences in the means of control and rPSGL-Ig–treated groups was performed with unpaired 2-tailed Student’s t tests and assuming normal distribution. Statistical significance was judged at P<0.05.

**Results**

**Time to Thrombolysis and Reocclusion**

The first set of experiments was performed with a group of animals that received rPSGL-Ig 250 µg/kg and a corresponding control group that received the same volume of rPSGL-Ig formulation buffer (vehicle) just before tPA. In control pigs, lysis of the thrombus was achieved in 44.4±13.3 minutes (Figure 1A). In animals that were treated with rPSGL-Ig, flow was reestablished 70% faster, after only 13.3±5.0 minutes.
minutes (Figure 1A). On thrombolysis, blood flow through the artery was graded on a scale of 0 to 3. As shown in Figure 2, the time taken for reestablishment of near-normal flow (score 2 or 3) was 59% faster in rPSGL-Ig–treated pigs than in control pigs (23.8 ± 16.9 versus 57.5 ± 15.8 minutes). Near-normal flow was not achieved in 1 animal in each group. In addition, arteries in control pigs reoccluded in 13.8 ± 6.9 minutes after the end of tPA infusion for 8 of 9 pigs, whereas no reocclusion was observed in the 1-hour observation period after the end of tPA infusion in 8 of 9 pigs that received rPSGL-Ig 250 μg/kg in addition to tPA (Table 1). Interestingly, the 1 rPSGL-Ig–treated pig in which reestablishment of near-normal flow could not be achieved. A series of angiograms from a representative control and a pig treated with rPSGL-Ig 250 μg/kg is presented in Figure 3.

In the second set of experiments, the dose of rPSGL-Ig was increased to 500 μg/kg to see whether the characteristics of reestablished flow could be further improved, and a corresponding control group was studied. As shown in Figure 1B, lysis of the thrombus in pigs that received rPSGL-Ig was accelerated by 61% compared with control (18.0 ± 8.4 versus 46.0 ± 8.9 minutes; Figure 2). Time taken for reestablishment of near-normal flow (score 2 or 3) was 58% faster in rPSGL-Ig–treated pigs than in control pigs (26.0 ± 20.7 versus 62.5 ± 22.2 minutes). Near-normal flow was not achieved in 1 animal in the control group. Again, arteries in control animals reoccluded in 6.0 ± 15.2 minutes after the end of tPA infusion in all 5 pigs in the control group (Table 1). Reocclusion did not occur in the 1-hour observation period in any of the 5 animals that received rPSGL-Ig.

The response in pigs treated with rPSGL-Ig 500 μg/kg was not significantly different from that in animals that received rPSGL-Ig 250 μg/kg in terms of acceleration of thrombolysis (61% versus 70%; P = 0.21), incidence of reocclusion (1 of 9 versus 0 of 5), and time taken for reestablishment of near-normal flow (58% versus 59% faster; P = 0.83). However, both groups of rPSGL-Ig–treated animals were significantly better than their respective controls.

Systemic Levels of Coagulation/Fibrinolytic Factors

Compared with baseline, high levels of tPA antigen and tPA activity were measured in pigs after tPA administration, and the levels were comparable in all control and rPSGL-Ig–treated groups. Selected results are shown in Table 2. The measures for the 2 control groups have been combined. No differences were detected between control and rPSGL-Ig–treated groups or between the 2 rPSGL-Ig groups. Plasma activated partial thromboplastin time increased in all groups on administration of heparin. Fibrinogen reduction was comparable in all groups, probably in relation to tPA administra-

**TABLE 1. Reocclusion of Arteries After Thrombolysis With tPA**

<table>
<thead>
<tr>
<th>Group</th>
<th>Incidence of Reocclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>8/9</td>
</tr>
<tr>
<td>rPSGL-Ig 250 μg/kg</td>
<td>1/8</td>
</tr>
<tr>
<td>Vehicle</td>
<td>5/5</td>
</tr>
<tr>
<td>rPSGL-Ig 500 μg/kg</td>
<td>0/5</td>
</tr>
</tbody>
</table>

Concurrent treatment with rPSGL-Ig prevents reocclusion after thrombolysis, despite the presence of the copper coil, vs control (P = 0.05). There was no statistical difference between the 2 rPSGL-Ig groups.
tion. Some reduction in $\alpha_2$-antiplasmin was seen in all groups. No changes in PAI-1 antigen levels occurred. These data suggest that rPSGL-Ig acts locally at the thrombus site and is not likely to adversely affect the endogenous coagulation/fibrinolytic system.

Serum Concentrations of rPSGL-Ig

Serum concentrations of rPSGL-Ig were measured in samples from rPSGL-Ig–treated pigs (Table 3). As evidence of the long half-life of this compound, its concentrations remain steady throughout the experiment.

Discussion

Thrombolytic agents have been key in significantly advancing therapy of myocardial infarction; however, their benefits have been limited by the ability of currently available thrombolytic regimens to promptly restore optimal myocardial reperfusion in only $\approx 50\%$ of patients.\textsuperscript{3} These patients are those for whom grade 3 flow, as described by the TIMI study group, is achieved within 90 minutes of treatment administration.\textsuperscript{26} Of these, half of the patients go on to experience recurrent ischemia (intermittent patency) or complete vascular reocclusion within hours to days.\textsuperscript{3,7} Failure to reestablish adequate coronary tissue perfusion is associated with a doubling in 30-day mortality, from 4\% to 8\%.\textsuperscript{27}

Thrombosis/thrombolysis is a dynamic system in which continuing platelet activation accompanied by the release of thrombin, fibrinogen, and PAI-1 at the thrombus site leads to ongoing thrombosis, which is directly linked to vascular reocclusion. In an effort to improve currently available thrombolytic therapy, the design of better thrombolytic agents, such as variants of tPA, and the use of concurrent thrombolytic therapy are being pursued. In this regard, inhibitors of platelet $\alpha_{\text{IIb}\beta_3}$ receptors have recently received

Figure 3. Angiograms at various times are presented for representative control and rPSGL-Ig 250 $\mu$g/kg pigs. Right internal iliac artery of control pig at (A) baseline, (B) occlusion, (C) onset of flow, and (D) termination on reocclusion is shown. In pig treated with rPSGL-Ig, angiograms taken at baseline (E) and at occlusion (F) were comparable to control. G, Onset of blood flow after lysis was accelerated; H, at termination, flow was still normal and area was well perfused.
much attention and have been successful in the clinic.⁹ The use of thrombolytic agents carries some risk, such as hemorrhage, which is potentiated on their combination with α₉β₃ inhibitors; hence, caution must be exercised.²⁸,²⁹

Because ongoing interactions of activated platelets with leukocytes via P-selectin influence fibrin deposition at the thrombus site,¹⁹ we investigated whether inhibition of these interactions would enhance the effect of thrombolytic therapy. We assessed the effect of using rPSGL-Ig (P-selectin antagonist) as an adjuvant to the thrombolytic agent tPA in a porcine model of copper coil–induced thrombosis. The thrombus formed within an intraluminal copper coil is believed to be fibrin-rich, consisting of adherent platelets bridged by a fibrin mesh and interspersed with erythrocytes.³⁰ Such thrombi have been shown to lyse on thrombolytic therapy, yet reocclusion is frequent. In fact, an α₉β₃ antagonist (RGDY) was not effective in preventing reocclusion of fibrin-rich thrombi.³⁰ Continuing platelet activation and fibrin accretion at the site of vascular injury have previously been shown to precipitate reocclusion.³¹ In this study, we show that treatment with rPSGL-Ig at the time of thrombolytic therapy accelerates thrombolysis and reestablishment of near-normal flow and also prevents subsequent reocclusion despite the continuing presence of the thrombogenic copper coil.

Platelets specifically recruit monocytes and neutrophils via their surface expression of P-selectin for thrombus stabilization and amplification.³² The release of tissue factor can occur on platelet-monocyte adhesion via P-selectin²⁰ or on activated monocyte adhesion to endothelium or fibrinogen via α₉β₃ (CD11b/CD18).³³,³⁴ Platelet-leukocyte interaction leading to tissue factor release and subsequent fibrin deposition was found to be P-selectin–dependent.¹⁹ In addition, thrombi formed in the presence of a P-selectin antibody lyse more rapidly on pharmacological thrombolysis, probably because of their reduced fibrin content.²² The present work shows that rPSGL-Ig, a form of the natural ligand for P-selectin, administered at the time of thrombolytic therapy enhances thrombolysis, most likely by inhibiting the continuing adhesion of platelets to leukocytes, leading to fibrin accretion. The colocalization of platelets and leukocytes in blood vessels and possible functional consequences of this interaction have also been reported in states such as myocardial infarction¹⁵ and unstable angina³⁶ and after angioplasty.³⁷,³⁸

rPSGL-Ig appears to shift the balance in favor of thrombolysis by acting locally at the thrombus site, because no differences in systemic coagulation/fibrinolytic parameters were detected. Therefore, P-selectin antagonism is likely
This work provides evidence that P-selectin antagonism is an effective approach to enhancing the effect of thrombolytic therapy. The physiological P-selectin ligand used in this work is a compound with a long half-life that has the added benefit of being administered as a bolus. rPSGL-Ig may represent a novel and safe therapeutic that could be important in accelerating thrombolysis, in promptly achieving optimal reperfusion, and in significantly reducing the incidence of acute reocclusion. Further clinical investigation of this compound is warranted.

Acknowledgments

The authors wish to thank Robin Shearer and Kathryn Madden, Charles River PharmServices, for technical assistance. We are grateful to Dr Yahye Merhi, Montreal Heart Institute, for porcine platelet-neutrophil binding assays. We thank Dr Richard Rockar (Laboratory Animal Resources) for his input, Annamaria Paone (Assay Systems) for rPSGL-Ig measurements, Gregg Timony (Pharmacokinetics) for pharmacokinetic assessment of rPSGL-Ig, and Kristin Murray (Bioanalytical Sciences, Genetics Institute) for determining binding to selectins of rPSGL-Ig. Finally, we thank the PSGL-1 team at Genetics Institute, Inc.

References


### TABLE 2. Plasma Measures of Coagulation/Fibrinolysis

<table>
<thead>
<tr>
<th>Test, Time Point</th>
<th>Control</th>
<th>rPSGL-Ig 250 µg/kg</th>
<th>rPSGL-Ig 500 µg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>aPTT, s</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>9.9 ± 4.1</td>
<td>11.5 ± 1.0</td>
<td>12.8 ± 2.9</td>
</tr>
<tr>
<td>Pre-tPA</td>
<td>142.5 ± 81.1</td>
<td>120.6 ± 40.3</td>
<td>174.4 ± 91.4</td>
</tr>
<tr>
<td>At flow</td>
<td>214.3 ± 104.1</td>
<td>154.6 ± 89.3</td>
<td>138.8 ± 41.3</td>
</tr>
<tr>
<td>Termination</td>
<td>242.4 ± 94.0</td>
<td>240.9 ± 81.3</td>
<td>236.4 ± 87.4</td>
</tr>
<tr>
<td>FG, mg/dL</td>
<td>189.6 ± 29.4</td>
<td>199.4 ± 51.5</td>
<td>176.0 ± 22.4</td>
</tr>
<tr>
<td>Pre-tPA</td>
<td>173.1 ± 20.8</td>
<td>180.6 ± 50.7</td>
<td>161.8 ± 12.8</td>
</tr>
<tr>
<td>At flow</td>
<td>167.6 ± 23.5</td>
<td>176.6 ± 49.8</td>
<td>157.8 ± 12.3</td>
</tr>
<tr>
<td>Termination</td>
<td>163.8 ± 22.0</td>
<td>171.6 ± 43.4</td>
<td>158.6 ± 14.1</td>
</tr>
<tr>
<td>α2-AP, %</td>
<td>91.1 ± 9.2</td>
<td>90.2 ± 6.0</td>
<td>89.0 ± 9.1</td>
</tr>
<tr>
<td>Pre-tPA</td>
<td>82.4 ± 8.4</td>
<td>82.6 ± 6.4</td>
<td>80.6 ± 3.6</td>
</tr>
<tr>
<td>At flow</td>
<td>75.2 ± 6.0</td>
<td>77.6 ± 6.4</td>
<td>77.2 ± 4.2</td>
</tr>
<tr>
<td>Termination</td>
<td>72.7 ± 6.2</td>
<td>72.8 ± 4.4</td>
<td>76.0 ± 3.1</td>
</tr>
<tr>
<td>PAI-1, ng/mL</td>
<td>8.4 ± 3.2</td>
<td>4.7 ± 2.1</td>
<td>4.4 ± 5.5</td>
</tr>
<tr>
<td>Baseline</td>
<td>9.3 ± 4.0</td>
<td>9.5 ± 2.2</td>
<td>3.6 ± 3.6</td>
</tr>
<tr>
<td>At flow</td>
<td>11.8 ± 7.0</td>
<td>6.1 ± 5.4</td>
<td>5.1 ± 4.2</td>
</tr>
<tr>
<td>Termination</td>
<td>11.0 ± 4.0</td>
<td>6.1 ± 5.3</td>
<td>6.0 ± 3.1</td>
</tr>
</tbody>
</table>

aPTT indicates activated partial thromboplastin time; FG, fibrinogen; α2-AP, α2-antiplasmin; and PAI-1, plasminogen activator inhibitor-1. There were no differences in systemic levels between the 2 groups of rPSGL-Ig–treated and control pigs (average of 2 groups).

### TABLE 3. Serum Levels of rPSGL-Ig

<table>
<thead>
<tr>
<th>Group</th>
<th>1 min After Dosing</th>
<th>At Termination</th>
</tr>
</thead>
<tbody>
<tr>
<td>rPSGL-Ig 250 µg/kg, µg/mL</td>
<td>3.0 ± 0.3</td>
<td>2.9 ± 0.5</td>
</tr>
<tr>
<td>rPSGL-Ig 500 µg/kg, µg/mL</td>
<td>6.9 ± 0.8</td>
<td>5.3 ± 0.6</td>
</tr>
</tbody>
</table>

Serum concentrations of rPSGL-Ig in rPSGL-Ig–treated pigs after bolus administration and at termination of the experiment.


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